IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Hadlaczky et al.
Serial No. : 09/724,726
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Examiner : Helmer, G.L.
Cust No. : 20985
Title : *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Gyula Hadlaczky declare as follows:

1) I am a co-inventor of the above-identified U.S. Patent Application Serial No. 09/724,726.

2) I have read the accompanying Declaration of Dr. Steven F. Fabijanski, which demonstrates the generation of SATACs in plants using the methods as taught in the application. This Declaration is incorporated herein by reference.

3) I am currently a Professor and Scientific Advisor at the Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences in Szeged, Hungary. I have held these positions since April 1993. Prior to that time, I held the positions of Senior Researcher (March 1983 – April 1993), Researcher (June 1980 – March 1983), and Assistant Researcher (February 1973 – June 1980) all at the Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences. I also am a cofounder of Chromos Molecular, Inc., a now-public Canadian company, which was founded to exploit the satellite artificial chromosomes (designated SATACs in the application; and presently referred to as Aces) described in the application. Chromos Molecular, Inc. has successfully commercialized the SATACs, including the spin-out of a subsidiary directed to plant applications of SATACs.

4) I received a Certificated Engineer degree from the University of Agricultural Sciences, Gödöllô, Hungary in 1972. I received University Doctor degree in cytogenetics from the University of Agricultural Sciences, Gödöllô, Hungary in 1974, and a Ph.D. degree in experimental biology from the Hungarian Academy of Sciences, Hungary in 1978. I

received D.Sci. in Biology from the Hungarian Academy of Sciences, Hungary in 1993. I have over 35 years experience working in the fields of chromosome and artificial chromosome technology, including the generation and uses of artificial chromosomes. I have authored or co-authored over 65 publications, and I have presented over 100 lectures in these fields. I also have received numerous awards and honors for my Scientific achievements including the distinction of Honorary Professor of the Department of Biotechnology at Gödöllô University; recipient of the Research Award of the Hungarian Academy of Sciences in 1974; recipient of the Academic Award for Young Scientists of the Hungarian Academy of Sciences in 1976; and recipient of the "Straub F. Bruno" Award from the Biological Research Center of the Hungarian Academy of Sciences in 1991. I also was the recipient of the Széchenyi award in 2000 for the work that is the subject of the above-captioned application.

5) The above-captioned application describes the generation of and preparation of satellite artificial chromosomes (designated SATACs in the application; and presently referred to as Aces). As provided in the application, this method is generally applicable to eukaryotic species to produce SATACs in any such species. The process by which SATACs are generated is a universal process, fundamental to replication and recombination in cells. As described in the above-captioned application, introduction of nucleic acid into the heterochromatic pericentric region of the chromosome, whether by targeted introduction or random introduction, initiates amplification events, leading to the generation of the *de novo* centromere, and ultimately of a SATAC. The underlying process of generating SATACs, including an amplification event and generation of a *de novo* centromere, is based on a universal mechanism shared by all species and cell types. This is described in the above-captioned application. As evidence of the universality of the process, this Declaration, and the accompanying Declaration of Fabijanski, describe the generation of SATACs in such diverse species as mammals, including rodents and human, and also in plants.

6) Using methods and materials described in the above-referenced application, and standard methods as described herein, myself and other scientists involved in these projects have demonstrated the generation of plant and human SATACs by the introduction of heterologous DNA into plant or human/hamster hybrid cells, respectively, leading to the amplification event that results in the generation of SATACs. This Declaration, and the accompanying declaration of Steven F. Fabijanski demonstrate the generation of SATACs in human/hamster hybrid cells and plants, respectively. The SATACS are generated as taught in the specification. Analysis of the resulting plant and human SATACs show that, as

taught in the application, plant and human satellite artificial chromosomes share the same identifying characteristics as mouse satellite artificial chromosomes, which are specifically exemplified in the subject application. Hence the methods used to generate SATACs in rodents is reproducible and can be applied to other species and also in other host cells. Therefore, as described in the above-captioned application, the underlying processes that result in preparation of SATACs are universally shared among species. Based upon the teachings in this application, SATACs can be generated in any selected species.

A description of the above-referenced methods and results follows.

I. MATERIALS AND METHODS

Generation of Human Artificial Chromosomes (Human SATACs)

1. Transfection of the 94.3 Cell line with Foreign DNA

a. The 94.3 Cell Line

A human/rodent somatic cell hybrid cell line, referred to as 94-3 (Repository No. GM10664), was obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Mutant Cell Repository (Camden, NJ). This cell line was generated by PEG fusion of human lymphoblasts with the HPRT-deficient Chinese Hamster cell line RJK88. The hybrid cells contain a translocated human chromosome 15 (the human der(15)t(X;15)) and human chromosome 22. The cells were cultured in DMEM medium containing 10% FBS.

The presence of human chromosomes in 94-3 cells was confirmed by *in situ* hybridization using biotin-labeled human genomic DNA probes and biotin-labeled human alpha satellite DNA probes. The hybridized probes were detected with FITC-conjugated avidin and with biotinylated anti-avidin. The chromosomes were counterstained with propidium iodide. The results of this analysis revealed that greater than 80% of the cells carried one or two human chromosomes.

b. Transfection of 94-3 Cells

Semiconfluent dishes (5 x 10^6 cells) of 94-3 cells were cotransfected with a mixture of ~1 μg pBabe Puro, ~2 μg pCH110 and ~70 μg pK161 using a calcium phosphate DNA precipitation method (Pharmacia).

i. pBabe Puro

pBabe Puro is a bacterial plasmid construct that contains DNA encoding the ampicillin resistance gene, the pUC origin of replication, and the puromycin N-acetyl transferase gene under control of the SV40 early promoter.

ii. pCH110

Plasmid pCH110 (Pharmacia) contains a β-galactosidase gene.

iii. pK161

Plasmid pK161 was generated by ligation of a 9-kb coding sequence of a mouse ribosomal RNA-encoding gene (*i.e.*, rDNA) with the cosmid vector pWE15 (Stratagene, La Jolla, California). The rDNA was obtained from megachromosomes that had been isolated from 1B3 cells (which were generated by repeated BrdU treatment and single cell cloning of H1xHE41 cells as described in the above-captioned application on page 30, lines 2-9, and page 71, lines 14-25, and which contain a truncated megachromosome) using fluorescence-activated cell sorting methods as described in the above-referenced application. The 9-kb rDNA fragment was isolated as follows. Following separation of the megachromosomes from the endogenous chromosomes in 1B3 cells, the isolated megachromosomes were stored in GH buffer (100 mM glycine, 1% hexylene glycol, pH 8.4-8.6 adjusted with saturated calcium hydroxide solution) and centrifuged into an agarose bed in 0.5 M EDTA. The isolated megachromosomes were cleaved with *Not*I, a rare cutting restriction endonuclease with an 8-bp GC recognition site. Fragments of the megachromosome were inserted into plasmid pWE15 (Stratagene, La Jolla, California) as follows. Half of a 100-µl low melting point agarose block (mega-plug) containing the isolated megachromosomes was digested with *Not*I overnight at 37°C. Plasmid pWE15 was similarly digested with *Not*I overnight. The mega-plug was then melted and mixed with the digested plasmid, ligation buffer and T4 ligase. Ligation was conducted at 16°C overnight. Bacterial DH5α cells were transformed with the ligation product and transformed cells were plated onto LB/Amp plates. Fifteen to twenty colonies were grown on each plate for a total of 189 colonies. Plasmid DNA was isolated from colonies that survived growth on LB/Amp medium and was analyzed by Southern blot hybridization for the presence of DNA that hybridized to a pUC19 probe. This screening methodology assured that all clones, even clones lacking an insert but containing the pWE15 plasmid, would be detected. All colonies were positive for hybridizing DNA.

Liquid cultures of all 189 transformants were used to generate cosmid minipreps for analysis of restriction sites within the insert DNA. Six of the original 189 cosmid clones contained an insert. One of these clones was designated pK161 (~9-kb insert). Portions of the sequence of the insert in pK161 were determined using an ABI sequencer and the dye-terminator cycle protocol.

A comparison of the sequence data to sequences in the GENBANK database revealed that the insert of pK161 corresponds to an internal section of the mouse ribosomal RNA gene (rDNA) repeat unit between positions 7551-15670 as set forth in GENBANK accession no. X82564. The sequence of the insert in pK161 diverges in some positions from the sequence presented in positions 7551-15670 of GENBANK accession no. X82564. Such divergence may be attributable to random mutations between repeat units of rDNA.

2. Analysis of Transfectants

Forty-eight hours after transfection of 94-3 cells with foreign DNA, the cells were exposed to 10μg/ml puromycin (Sigma). Sixty-eight individual puromycin-resistant colonies were selected and propagated and then analyzed for the presence of artificial chromosomes by Southern hybridization, LacZ staining, C-banding and *in situ* hybridization.

II. Results

The results demonstrate that human artificial chromosomes (human SATACs) can be generated in human cells.

Generation of a Human SATAC

1. Selection and Screening for Amplification

Southern hybridization of DNA isolated from the selected 94-3 cell transfectants was used as a primary screen to detect cells containing chromosomes that had undergone amplification of the pericentric DNA indicative of satellite artificial chromosome formation. DNA purified from the transfectants was digested with EcoRI and hybridized with pBabe Puro. DNA from more than 40% of the 68 selected transfectants showed hybridization at levels indicative of high-copy numbers of integrated pBabe Puro sequences. This result correlates with an amplification of the integrated pBabe Puro DNA. Transfectants were also analyzed for expression of β-galactosidase from the integrated pCH110 DNA by using standard LacZ-staining techniques. Twenty-one of the 68 transfectants showed β-galactosidase expression detectable in this assay.

Amplification also was assessed by cytological analysis of the transfectant by C-banding according to the Giemsa/barium hydroxide method (see *e.g.*, Sumner (1972) *Exp. Cell Res.*, 75:304-306). This staining method specifically detects constitutive heterochromatin. Thirty percent of the transfectants showed amplified heterochromatic segments through C-banding. Because Chinese hamster cell chromosomes do not contain any large constitutive heterochromatic regions, whereas human chromosomes 15 and 22 do have somewhat larger regions of constitutive heterochromatin, the presence of chromosomes

containing extensive regions that are readily detectable by C-banding supports the conclusion that amplification of the heterochromatin had most likely occurred in the human chromosomes of many of the transfectants.

2. Detection and Characterization of Artificial Chromosomes

a. C-banding

One of the transfected clones (clone 23) was analyzed further. C-banding of clone 23 revealed the presence of a sausage chromosome with the characteristic extended heterochromatic arm. A human satellite artificial chromosome that is ~100-120 Mb in size also was detected in clone 23. C-banding of the chromosomes in clone 23 cells revealed that the human satellite artificial chromosome resulted from further amplification of the heterochromatic arm of a sausage chromosome. Both arms of the human artificial chromosome stained as predominantly heterochromatic, in contrast to a sausage chromosome in which only one arm stains in C-banding. The presence of two heterochromatic arms indicates that a sausage chromosome had undergone amplification, including a centromere duplication, leading to the formation of another heterochromatic chromosome arm and the generation of a satellite artificial chromosome.

b. *In Situ* Hybridization

Clone 23 also was subjected to *in situ* hybridization with corresponding propidium iodide staining to counterstain the chromosome structure. Hybridization with a biotinylated probe containing human genomic DNA from the EJ30 cell line revealed that the site of hybridization of the probe was on the chromosome containing the heterochromatic arm (*i.e.* sausage chromosome). Thus, it was possible to conclude that the heterochromatic arm was formed on a human chromosome. Further analysis with a biotinylated human alpha satellite DNA probe confirmed that the heterochromatic arm was formed on a human chromosome, and *in situ* hybridization with a biotin-labeled human chromosome 15-specific alpha satellite DNA probe (Oncor catalog no. P5034 D15Z1) revealed that the saugage chromosome derived from human chromosome 15. *In situ* hybridization of clone 23 with pBabe Puro and rDNA (contained in pK161) probes revealed a hybridization signal only in the heterochromatic arm of the sausage chromosome, indicating that these genes co-amplified in the heterochromatic arm of the sausage chromsome.

Analysis of clone 23 cells by hybridization to biotin-labeled human alpha satellite DNA and biotin-labeled pBabe Puro also was used in characterization of the human satellite artificial chromosome. Because the hybridization of the two different probes was to the same chromosome, these results demonstrate that the artificial chromosome contains the intergrated foreign DNA and is human in origin. Additionally, *in situ* hybridization of clone 23 cells with a biotin-labeled human chromosome 15-specific alpha satellite DNA probe (Oncor catalog no. P5034 D15Z1) revealed that the satellite artificial chromosome derived from human chromosome 15.

III. CONCLUSION

The above experiments demonstrate that the SATAC's are generated by a fundamental and universal process. This is evidenced by the above data, and the data in the accompanying Declaration of Fabijanski. The data demonstrate the preparation of SATACs in such diverse species as human and plant cells. The results show that the generation of a plant or human SATAC is induced by the integration of exogenous DNA molecules into the pericentric regions of the native chromosome, which leads to a large-scale amplification and *de novo* formation of a stable, heterochromatic SATAC. Thus, the inducible replication-directed large scale amplification and *de novo* chromosome formations are general across diverse species.

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Serial No. : 09/724,726
Declaration

Attorney's Docket No.: 17084-004006/402E

I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeapardize the validity of the application or any patent resulting therefrom.

May 28, 2007
Date

Gyula Hadlaczky